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A role for β,β-xanthophylls in Arabidopsis UV-B photoprotection

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Running title: β,β-xanthophylls protect Arabidopsis against UV-B damage

Highlight

We demonstrate that carotenoids have a role in UV-B photoprotection in *Arabidopsis thaliana*. Particularly, *we* show that β,β-xanthophylls, but not other plastidial isoprenoids, are required for the UV-B protection observed.

Abstract

Plastidial isoprenoids, such as carotenoids and tocopherols, are relevant anti-oxidant metabolites synthesized in plastids from precursors generated by the methylerythritol 4-phosphate (MEP) pathway. In this work, we found that irradiation of *Arabidopsis thaliana* plants with UV-B caused a strong increase in the accumulation of the photoprotective xanthophyll zeaxanthin but also slightly higher levels of γ-tocopherol. Plants deficient in MEP enzymes 1-deoxy-D-xylulose 5-phosphate synthase and 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase showed a general reduction in both carotenoids and tocopherols associated with increased DNA damage and decreased photosynthesis after UV-B exposure. Genetic blockage of tocopherol biosynthesis did not affect DNA damage accumulation. In contrast, *lut2* mutants accumulating β,β-xanthophylls showed decreased DNA damage when irradiated with UV-B. Analysis of *aba2* mutants showed that UV-B protection is not mediated by ABA (a hormone derived from β,β-xanthophylls). Plants accumulating β,β-xanthophylls also show decreased oxidative damage and increased expression of DNA repair enzymes, suggesting that this can be a mechanism for these plants to decrease DNA damage. In addition, *in vitro* experiments also provide evidence that β,β-xanthophylls can directly protect against DNA damage by absorbing radiation. Together, our results suggest that xanthophyll cycle carotenoids that protect against excess illumination could also contribute to the protection against UV-B.

Key words: isoprenoids, MEP pathway, UV-B damage, violaxanthin, xanthophylls, zeaxanthin.

Introduction

In natural environments, plants are continuously exposed to different potentially damaging conditions. Sunlight reaching the earth's surface is composed by UV-B (280-315 nm), UV-A (315-400 nm), photosynthetically active (PAR, 400-700 nm) and infrared radiation (>700 nm). In recent decades, UV-B radiation levels reaching the earth have increased due to a decrease in the ozone layer as a result of the use of chlorofluorocarbons (McKenzie *et al.*, 2007). Despite the use of these compounds was forbidden in the Montreal Protocol, the problem still continues possibly because of the release of greenhouse gasses (Newman *et al.*, 2001). In this way, sunlight is required for photosynthesis and plant development, but it also represents a major hazard. For example, too much light energy can overwhelm the photosynthetic capacity of the cell and eventually cause photooxidative damage. Also, excess UV-B levels can produce mutations in the DNA. Thus, plants, due to their sessile conditions, have evolved different adaptation strategies to protect themselves against excessive PAR or UV-B levels (Li *et al*., 1993; Stapleton and Walbot 1994; Landry *et al.*, 1995; Jansen *et al.*, 1998; Wargent *et al.*, 2011; Agati *et al.*, 2013; Solovchenko and Neverov, 2017; Baker *et al.*, 2017). In particular, carotenoids have been directly linked to photoprotection of the photosynthetic apparatus in plants. This ability is probably due to their function as efficient quenchers of high energy shortwave radiation. Interestingly, xanthophylls, a term that includes all the oxygenated derivates of carotenes, absorb the shortest wavelength radiation within the light harvesting complexes (Middleton and Teramura, 1993).

Among the several UV-B protection mechanisms that plants have developed, one of the most studied is protection by secondary metabolism pathways products, in particular the production of phenolic compounds in leaves (Li *et al*., 1993; Stapleton and Walbot 1994; Landry *et al.*, 1995). Because DNA strongly absorbs UV-B; it is one of the most important targets of UV-B induced damage (Britt, 1996). Phenolic compounds have been demonstrated to decrease UV-B induced DNA damage. For example, in maize, plants with increased flavonoid levels (primarily anthocyanins), have lower DNA damage after UV-B exposure than plants that are genetically deficient in these compounds (Stapleton and Walbot, 1994). Moreover, we have recently demonstrated that flavonols, a different class of flavonoids, are able to protect Arabidopsis plants from damage generated by exposure to UV-B radiation, including in the DNA (Emiliani *et al.*, 2013).

In addition to flavonoids, recent metabolomics studies have suggested that isoprenoids could also participate in UV protection in plants (Matus, 2016). Isoprenoids are a

group of different biologically active compounds that participate in a wide range of biological functions including photosynthesis, respiration, growth, defense, and adaptation to environmental conditions. Examples of isoprenoids are the photosynthetic pigments chlorophylls and carotenoids, including the xanthophylls; the hormones abscisic acid (ABA), gibberellins, cytokinins and brassinosteroids; and tocopherols (**Fig. 1A**; Vranová *et al.*, 2013). Among them, carotenoids and tocopherols protect chloroplasts against high light, either by dissipating excess excitation energy as heat or by scavenging reactive oxygen species (ROS) and suppressing lipid peroxidation (Peñuelas and Munné-Bosch, 2005). All isoprenoids are derived by consecutive condensations of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In plant plastids, both IPP and DMAPP are produced by the methylerythritol 4-phosphate (MEP) pathway (**Fig. 1A**; Rodríguez-Concepción and Boronat, 2015). While the effect of flavonoids on filtering or absorbing UV-B is clear, the role of isoprenoid production in UV-B protection has not been clearly demonstrated. Therefore, to further expand the identification of plant metabolites with adaptive functions to allow survival under conditions of increased UV-B radiation, we analyzed the role of different isoprenoids from the MEP pathway in UV-B protection. For this aim, *Arabidopsis thaliana* transgenic and mutant lines deficient in the expression of different enzymes of the synthesis of isoprenoid derivatives from the MEP pathway were used. DNA damage accumulation, photosynthesis, oxidative stress and isoprenoid accumulation patterns parameters were analyzed and compared using wild type (WT) and transgenic/mutant plants. The results presented here demonstrate that MEP derivate isoprenoids, and in particular carotenoids of the β,β-xanthophyll type, protect plants against UV-B induced damage.

Materials and methods

Plant material, growth conditions and UV-B treatment

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) lines were used in the experiments. The list of mutants and transgenic lines used is presented in **Table S1**. Arabidopsis plants were sown directly on soil and placed at 4ºC in the dark, and were then transferred and grown in a growth chamber at 22°C with supplemental visible light (100 µE m^{-2} s^{-1}) with a 16-h / 8-h light/dark regime. After four weeks, a group of plants were exposed to UV-B radiation for 4-h using UV-B lamps mounted 30 cm above the plants (Bio-Rad, Hercules, California) at a UV-B intensity of 2 W m^{-2} and a UV-A intensity of 0.7 W m^{-2}. The bulbs were covered with cellulose acetate filters (100 mm extra clear cellulose acetate plastic, Tap Plastics, Mountain View, CA) to exclude wavelengths lower than 290 nm. The lamps

used have emission spectra from 290-310 nm, and a peak at 302 nm. A UV spectrum from a similar set up was previously shown in Casati and Walbot (2003). A different group of control plants were treated with the same bulbs covered with a polyester film (100 µm clear polyester plastic; Tap Plastics, Mountain View, CA) that absorbed most UV-B radiation from the lamps (UV-B: 0.04 W m^{-2}; UV-A: 0.4 W m^{-2}). None of the plants used showed any visible phenotype after the UV-B treatments. Leaf samples were collected immediately after the treatments and stored at -80ºC. Experiments were repeated at least three times.

Soluble phenolic extraction and quantification

For measurements of UV-absorbing phenolic pigments, 100 mg of frozen plant samples in liquid nitrogen were ground to a powder with a mortar and pestle. The powder was extracted during 8 h with 0.6 mL of acidic methanol (1% [v/v] HCl in methanol), followed by a second extraction with 0.6 mL of chloroform and 0.3 mL of distilled water, as described in Emiliani *et al.* (2013). The extracts were vortexed, then centrifuged for 2 min at 3,000 g and the upper aqueous phases were collected. The final pH is nearly 1; however, flavonoids and other phenolic compounds that absorb UV radiation are stable at this pH (Friedman and Jurgens, 2000). The absorbance of the aqueous phase solution was measured at 312 nm.

Maximum efficiency of photosystem II measurements

Chlorophyll (Chl) fluorescence parameters were measured on dark-adapted leaves using a Qubit Systems pulse-modulated fluorometer (Qubit Systems Inc, Ontario, Canada). The minimum Chl fluorescence at an open photosystem II (PSII) center (Fo) was determined after 20 min in the dark using light (655 nm) at an intensity of 0.1 µmol m^{-2} s^{-1}. A saturation pulse of white light (2,500 µmol m^{-2} s^{-1} for 0.8 s) was applied to determine the maximum Chl fluorescence at closed PSII centers in the dark (Fm). The parameters of light-adapted leaves (Fm' and Ft) were measured after 20 min of illumination with 100 µmol m^{-2} s^{-1}. Maximum efficiency of PSII (Fv/Fm) and quantum yield of PSII (ϕ_{PSII}) was calculated as Fm – Fo/Fm and Fm' - Ft/Fm' (Baker and Rosenqvist, 2004; Ifuku et al., 2005). The measurements were made three times in at least three different plants.

DNA Damage Analysis

CPD levels were determined using monoclonal specific antibodies from Cosmo Bio Co., Ltd. (TDM-2; Japan) as described in Emiliani *et al.* (2013).

For in vitro DNA damage analysis, genomic DNA from *A. thaliana* leaves (2 µg) was incubated in triplicate with 14 µg mL^{-1} violaxanthin purified from mango (*Mangifera indica*), with 100 µg mL^{-1} rutin, or in the absence of any compound for 30 min at 4°C in a final volume of 100 µL before UV-B irradiation. Commercial quercetin-3-β-D-rutinoside (rutin, Sigma-Aldrich) was used as a positive control (100 µg mL^{-1}), as it protects against UV-B induced DNA damage (Kootstra, 1994). Samples were then irradiated at a UV-B intensity of 0.5 W m^{-2} and a UV-A intensity of 0.45 W m^{-2} in 96-well plates for 5, 10 and 15 min. Control samples were exposed for 15 min under the same UV-B lamps but covered with polyester filters to exclude UV-B radiation. After exposure, samples were transferred to tubes, genomic DNA was denatured in 0.3 N NaOH for 10 min and dot blotted in a nylon membrane for CPDs quantification as described above. The experiments were repeated at least three times.

Isoprenoids measurements and quantification

The isoprenoid pigment content of Arabidopsis leaf samples were examined by HPLC-DAD-FLD (Fraser *et al.*, 2000). All samples were protected from light and heat during all steps during the extraction procedure. 4 mg of lyophilized Arabidopsis tissue was extracted with methanol (400 µL) with the addition of 1.2 mg of canthaxanthin as an internal standard. The suspension was mixed by agitation for 10 min at 4°C and 400 µL of Tris-HCl (1 M, pH 7.5) was added, with further agitation at 4°C for 10 min. After that, chloroform (800 µL) was added and mixed for 5 min a 4°C. After centrifugation at maximum speed in a chilled benchtop microcentrifuge (Eppendorf 5418R, Hamburg, Germany) for 5 min at 4°C, two different phases were produced. The upper phase was removed with a Pasteur pipette, and the lower phase, corresponding to the organic layer, was dried in a vacuum centrifuge (Eppendorf Concentrator Plus). Dried residues were resuspended in 200 µl ethyl acetate and filtered through a 0.2 µm Polytetrafluoroethylene microfilter (Supelco). A 10 µL aliquot of each sample was injected onto an Agilent Technologies 1200 series HPLC system equipped with a diode array (Santa Clara, CA, USA). A C30 reverse-phase column (YMC Carotenoid, 250 x 4.6 mm x 3 µm) was used, with three mobile phases consisting of methanol (A),

water/methanol (20/80 v/v) containing 0.2% ammonium acetate (w/v) (B), and tert-methyl butyl ether (C). Metabolites were separated with the following gradient: 95% A, 5% B isocratically for 12 min, a step up to 80% A, 5% B, 15% C at 12 min, followed by a linear gradient up to 30% A, 5% B, 65% C by 30 min. The flow rate was maintained at 1 $mL \cdot min^{-1}$. Isoprenoid pigments were monitored at 472 and 650 nm and compared to the retention times of chlorophyll and β-carotene authentic standards. Tocopherol quantification was accomplished through fluorescence detection (excitation 290 nm, emission 330 nm) and comparison to a standard curve constructed with α-tocopherol. Peak areas of the standards were determined at the maximum absorbance wavelengths using the Waters Millennium32 software supplied. Peak areas of the standards were determined at the maximum absorbance wavelengths using the Waters Millennium32 software supplied.

Gene expression analyzes by RT-qPCR

Total RNA was isolated using 100 mg of tissue with the TRIzol reagent (Invitrogen, Carlsbad, CA) and was then treated with DNase (Promega, Madison, WI). RNA was converted into first-strand cDNA using oligo-dT as a primer with SuperScript II reverse transcriptase (Invitrogen). The cDNA was used as a template for quantitative PCR amplification in a MiniOPTICON2 apparatus (Bio-Rad), using SYBRGreen I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers were designed to generate unique 150-250 bp-fragments with the PRIMER3 software (Rozen and Skaletsky, 2000). A list of primers used is shown in **Table S2**. Three biological replicates were performed for each sample. To normalize the data of UV treatments, primers for *CPK3* were used. Amplification conditions were as follows: 2 min denaturation at 94°C; 40 to 45 cycles at 94°C for 10 s, 57°C for 15 s, and 72°C for 20 s, followed by 5 min at 72°C. Melting curves for each PCR product were determined by measuring the decrease of fluorescence with increasing temperature (from 65°C to 95°C).

Electrolyte leakage and thiobarbituric acid reactive substances content

Electrolyte leakage and thiobarbituric acid reactive substances content were determined as described in Emiliani *et al.* (2013).

Violaxanthin extraction and purification

The violaxanthin purification protocol was adapted from Araki *et al.* (2016) with some modifications. Two hundred grams of mango (*Mangifera indica*) from a fruit market cut into small pieces was suspended in 300 mL of saturated aqueous $NaHCO_3$, and was homogenized in a blender for 1 min. 500 mL of acetone was added to the mango solution, the mix was stirred for 5 min and then filtered using miracloth. The solid remaining on the filter paper was collected and mixed in 300 mL of CH_2Cl_2/acetone (2:1, v/v) for 15 min at room temperature to extract carotenoids. The mix was filtered again, but now the filtrate (600 mL) was collected and concentrated into a small volume to remove the CH_2Cl_2 and the acetone. 200 mL of EtOAc and 200 ml of H2O was added, without pH adjustment. The EtOAc layer was concentrated to dryness; and then, the carotenoid extract was saponified by the addition of 10 mL of a KOH solution (5 g of KOH/100 mL of 90% EtOH (v/v)) and 5 mL of CH_2Cl_2. After this, 200 mL of EtOAc and 200 mL of H_2O were added and mixed. The EtOAc layer was collected and concentrated to dryness, obtaining a red oil. The red oil was subjected to chromatography on a 20 × 20 cm aluminum plate of TLC silicagel 60 F254 (Merck) using hexane/acetone (3:1, v/v) as a mobile phase. Violoxanthin was separated as a yellow band with a rf = 0.35, and it was collected by carefully scraping the plate with a spatula. The compound was extracted from the silica with methanol. The solvent was filtered and evaporated, and purified violaxanthin was concentrated to dryness.

Statistical analysis

Data presented were analyzed using one-way and two-way analysis of variance (ANOVA); or by Student's t test. Minimum significant differences were calculated by the Bonferroni, Dunett, Turkey and Duncan tests ($P < 0.05$) using Sigma Stat 3.1 and Graphpad Prism 5.03 Software.

Factor Analysis was performed on data sets including average values of isoprenoids and CPD levels relative to those in Col-0 control plants. Multivariate statistical analysis was carried out using the software package XLSTAT (Microsoft Excel).

Results

Isoprenoid profile changes in Arabidopsis plants exposed to UV-B

To initially investigate a possible involvement of plastidial isoprenoids that derive from the MEP pathway in UV-B protection in Arabidopsis, we first analyzed plastidial isoprenoid profile changes after exposure of WT plants of the Columbia ecotype (Col-0) to UV-B for 4 hours. Control treatments were performed using a polyester screen that absorbed most UV-B radiation. While some isoprenoids remained unchanged after the UV-B treatment, a low although significant decrease in lutein, β-carotene and chlorophylls was detected by HPLC analysis (**Table 1**; **Table S3**). Moreover, a significant increase both in zeaxanthin (13-fold increase compared to control samples) and γ-tocopherol (1.88-fold increase**; Table 1**) were measured, suggesting that these particular plastidial isoprenoids could participate in UV-B protection in Arabidopsis plants. Interestingly, analysis of publicly transcriptomic data (GSE80111; Das *et al*, 2016) showed that some carotenoid biosynthetic genes were up-regulated in 4-week-old Arabidopsis plants 4h after UV-B exposure (1.31 $kJ.m^{-2}$; **Fig. S1**). In particular, the *BCH1* and *BCH2* genes, encoding enzymes involved in the production of zeaxanthin from β-carotene, was induced about 11-fold in UV-B irradiated plants. The gene encoding zeta-carotene epoxidase (*ZEP*), which transforms zeaxanthin into violaxanthin, was induced ca. 2-fold. The *VTE1* gene, involved in tocopherol biosynthesis, was similarly up-regulated (**Fig. S1**). To further validate if under our experimental conditions some of these transcripts were also UV-B regulated, we conducted qRT-PCR analysis using RNA samples extracted from plants irradiated under the same conditions as those used in HPLC analysis. Both *BCH1* and *BCH2* were significantly induced by UV-B in our assays (**Fig. 1b**), similarly as *PSY* and *ZDS*, which encode enzymes in the carotenoid, xanthophyll and carotene synthesis. However, neither *ZEP* nor *VDE*, which encodes the enzyme that converts violaxanthin into zeaxanthin, showed changes in their expression under our experimental conditions (**Fig. 1b**). Altogether, these results suggest that the observed changes in the isoprenoid profile of UV-B-exposed plants (i.e. higher zeaxanthin and γ-tocopherol accumulation but virtually unchanged levels of the rest of carotenoids, α-tocopherols or chlorophylls) might be mainly caused by changes in the levels and hence the activities of the enzymes that produce these particular metabolites or transform them into downstream products.

Arabidopsis plants with decreased plastidial isoprenoid levels have increased DNA damage after UV-B exposure

To investigate some of the *in vivo* roles of carotenoids and tocopherols in UV-B protection in plants, we used transgenic Arabidopsis plants with decreased activity of MEP pathway enzymes. We first used plants that express an antisense construct against the *DXS* transcript, (*A6;* Estévez *et al.*, 2001, **Table S1**), which encodes the MEP pathway entry enzyme 1-deoxy-D-xylulose 5-phosphate synthase. These plants that were generated in the RLD background have decreased levels of *DXS* transcripts and DXS protein; and they were previously demonstrated to have a general decrease in the plastidial isoprenoids (Estévez *et al.*, 2001, **Table S4**). The effect of reduced amount of isoprenoid products of the MEP pathway protecting DNA against damage after a 4h-UV-B treatment was first analyzed. WT (RLD) and Arabidopsis plants deficient in *DXS* expression were grown in the greenhouse under very low UV-B (0.04 W m^{-2}, control conditions) for 4 weeks, and plants were then exposed to UV-B radiation for 4h (2 W m^{-2}). As a control, different sets of plants were irradiated with the same lamps covered with a polyester plastic that absorbs UV-B (see Materials and methods). Leaf samples from control and UV-B-treated plants were collected immediately after the end of the treatment. DNA was extracted and cyclobutane pyrimidine dimer (CPD) abundance was compared by an immunological assay; this assay detects CPDs using monoclonal antibodies specifically raised against them. Comparison of CPD accumulation in samples from WT and transgenic plants after the 4h-UV-B treatment is shown in **Fig. 2A**. Under control conditions, the steady state levels of CPDs in WT and *DXS* deficient plants were similar (**Fig. 2A**). After the UV-B treatment, unrepaired lesions were accumulated in all plants; however, the accumulation of CPDs in *A6* plants was more severe than that in WT plants (**Fig. 2A**), suggesting that products of the MEP pathway protect plants against DNA damage by UV-B.

Decreased flux through the MEP pathway in DXS-deficient plants is expected to result in lower levels of the pathway intermediate methylerythritol cyclodiphosphate (MEcPP), a metabolite demonstrated to mediate responses to stress (Xiao *et al.*, 2012). To test whether the decreased protection against UV-B irradiation in *A6* plants was a consequence of reduced levels of MEcPP or resulted from a decrease production of downstream isoprenoids, we used Arabidopsis *constitutive subtilisin3 (csb3)* mutants (Flores-Perez *et al.,* 2008). Unlike DXS-defective lines, *csb3* plants show lower levels of MEP-derived plastidial isoprenoids but higher levels of MEcPP (Flores-Perez *et al.,* 2008; Gonzalez-Cabanelas *et al.*, 2015) due to reduced activity of the enzyme 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase (HDS),

which transforms MEcPP into downstream intermediates of the MEP pathway (**Fig. 1A**). As shown in **Fig. 2A**, *csb3* plants showed an increased DNA damage after UV-B irradiation compared to the Col-0 WT. The similar phenotype of DXS-defective and HDS-defective lines in terms of UV-B protection argues against a role of MEcPP in this response and supports the conclusion that MEP-derived isoprenoids are required for the protection.

Decreased levels of carotenoids and tocopherols also result in increased photosystem II damage in plants irradiated with UV-B

To further investigate the effect of decreased levels of products of the MEP pathway in photoprotection to the photosynthetic machinery after UV-B exposure, the maximum efficiency of photosystem II (PSII, Fv/Fm) was assessed immediately after a 4h-UV-B treatment using the same plant genotypes. Under control conditions, this photosynthetic parameter was similar in all plants analyzed (**Fig. 2B**). After the UV-B treatment, WT plants in both genetic backgrounds, and *A6* and *csb3* plants showed a decrease in the maximum efficiency of PSII (**Fig. 2B**); however, this decrease was significantly more pronounced in *A6* and *csb3* plants than in WT plants from the same genetic background. Interestingly, WT RLD and *A6* plants showed similar levels of UV-B absorbing phenolic pigments under control conditions and after the UV-B treatment, suggesting that compounds synthesized through the MEP pathway are required for UV-B protection in Arabidopsis besides phenolic compounds. Moreover, in the Col-0 background, the *csb3* mutants accumulate higher UV-B absorbing phenolic compounds levels than WT plants (**Fig. 2C**), again demonstrating a role of products of the MEP pathway in UV-B protection.

Tocopherols are not required for protection against UV-B exposure

To discriminate between the possible contribution of carotenoids and tocopherols to the protection against UV-B, we next carried out a genetic approach based on analyzing the protection against UV-B of mutants lacking one of the two groups of metabolites. Carotenoid-deficient mutants show an albino seedling phenotype and are unable to survive. By contrast, mutants devoid of tocopherols are green and viable. In particular, Arabidopsis *vte1* and *vte2* mutants are deficient in tocopherol cyclase and homogentisate phytyltransferase activities, respectively (**Fig. 1**), and hence lack tocopherols (Sattler *et al.,* 2003; Maeda *et al.,* 2008).

Both mutants showed CPD levels similar to WT plants after UV-B exposure (**Fig. 3**), supporting the conclusion that tocopherols do not have a major role in UV-B protection in Arabidopsis.

β,β-xanthophylls protect plants against DNA damage after UV-B exposure in Arabidopsis

To investigate whether particular carotenoids were more efficient in protecting plants against UV-B irradiation, we used Arabidopsis mutants defective in only some types of carotenoids. After desaturation and isomerization of phytoene to produce lycopene, the carotenoid pathway branches towards carotenes and xanthophylls with two β rings (such as β-carotene and derived β,β-xanthophylls) or with one β and one ε ring (such as lutein) (**Fig. 1A**). Loss of function of *LUT2*, the only lycopene ε-cyclase found in Arabidopsis (Ruiz-Sola and Rodriguez-Concepción, 2012), results in no accumulation of the β,ε-xanthophyll lutein, but in increased levels of the β,β-xanthophylls: violaxanthin, zeaxanthin and antheraxanthin (**Table 1**). Interestingly, *lut2* mutants showed lower DNA damage after UV-B exposure than WT plants (**Fig. 4A**), suggesting that one or several of the β,β-xanthophylls accumulated in these plants might have a role in UV-B protection in Arabidopsis.

Next, we assayed UV-B irradiation-triggered DNA damage in mutants defective in the *ABA1* gene, encoding the only zeaxanthin epoxidase (ZEP) enzyme found in Arabidopsis (Ruiz-Sola and Rodriguez-Concepción, 2012). ZEP transforms zeaxanthin into violaxanthin via antheraxanthin (**Fig. 1**) and hence *aba1* mutants do not accumulate violaxanthin or antheraxanthin but have highly increased levels of zeaxanthin compared to WT plants (**Table 1; Table S3**). Loss of violaxanthin and antheraxanthin, however, did not reduce the protection against UV-B in *aba1* mutants, as they showed similar CPD levels as WT plants after UV-B exposure (**Fig. 4B**). It is possible then that high zeaxanthin levels (which accumulate 438-fold higher in *aba1* than in WT plants; **Table 1**) could compensate for the violaxanthin and antheraxanthin deficiency.

To further confirm our hypothesis, we performed a Factor Analysis, which is a multivariate statistical method, with the aim of analyzing the pattern of relationships among isoprenoids levels and DNA damage. Factor analysis after varimax rotation indicates that the first two factors explain 68.91 % of total variance. In order to find the associations, factor-loading values were taken into account and those higher than 0.5 are considered significant. **Table S5** shows that the second factor is positively related to lutein, *γ*-tocopherol and DNA damage, while it is negatively related to violaxanthin and antheraxanthin. This factor shows

that DNA damage is associated to violaxanthin and its precursor antheraxanthin in a negative way; thus, this statistical analysis validates our hypothesis that these xanthophylls can protect Arabidopsis against DNA damage caused by UV-B radiation.

ABA is not involved in UV-B protection against DNA damage by UV-B in Arabidopsis

The described results show that β,β-xanthophylls contribute to the protection of Arabidopsis plants against UV-B exposure and suggest that zeaxanthin can partially compensate for the loss of violaxanthin and antheraxantin. As violaxanthin and derived neoxanthin can be used for the production of ABA through NCED enzymes activity (**Fig. 1A**), we next aimed to test whether this hormone could also participate in UV-B protection. To this end, UV-B irradiation experiments were conducted using *aba2* mutants (**Fig. 1A**), which show WT levels of carotenoids (including β,β-xanthophylls) but are deficient in ABA synthesis and accumulation (**Table 1,** González-Guzmán *et al.*, 2002). As shown in **Fig. 4B**, *aba2* mutants showed similar CPD accumulation as *aba1* and WT Col-0 plants, demonstrating that protection against UV-B damage is independent of ABA.

β,β-xanthophylls protect against oxidative damage after UV-B exposure in Arabidopsis

To better understand the role of β,β-xanthophylls in UV-B protection, we examined the expression of genes that participate in UV-B signaling and response in WT plants, in *cbs3* mutants (deficient in β,β-xanthophylls), and in *lut2* plants (accumulating β,β-xanthophylls). The expression of the UV-B photoreceptor *UVR8* was similar in all samples analyzed, independently of the light condition or the β,β-xanthophyll levels in the plants used (**Fig. 5A**). On the other hand, *HY5* (encoding a transcription factor that regulates UV-B responses), *CHS* and *F3H* (encoding chalcone synthase and flavanone 3-hydroxylase, respectively, that participate in the biosynthesis of flavonoids, UV-B absorbing phenolics in plants) were significantly increased after UV-B exposure in all plants (**Fig. 5B-D**). This was also true when transcript levels were analyzed in *aba1* and *aba2* mutants (**Fig. S2 A-C**). In *lut2*, *aba1* and *aba2* mutants, and in agreement with transcript expression analysis, UV-B absorbing phenolics also increased after exposure (**Fig. S2 D-E).** However, none of these changes correlated with the DNA damage accumulated after UV-B exposure in the mutants analyzed (**Fig. 2 and 4**). Consequently, DNA damage protection by UV-B mediated by β,β-xanthophylls

is not through changes in the expression of UV-B signaling and response genes or changes in the levels of UV-B absorbing phenolics.

On the other hand, transcript levels of the DNA repair enzymes *UVR2* (encoding a CPD photolyase) and *UVR7* (encoding ERCC1, a DNA excision repair protein) were significantly and higher increased after UV-B exposure in *lut2* than in WT plants, in agreement with the decreased CPD accumulation measured in these plants (**Fig. 4 and 5**). In *cbs3* mutants, while *UVR2* levels were similar to those in WT plants after UV-B exposure, *UVR7* was lower expressed after irradiation, also in agreement with DNA damage measured (**Fig. 2 and 5**). Thus, β,β-xanthophyll levels may affect the expression of DNA repair enzymes, and, as a consequence, DNA damage accumulation after UV-B.

In plants, carotenoids including xanthophylls, are considered to be the first line of protection against singlet oxygen stress (Ramel *et al.*, 2012).Therefore, we analyzed if changes in transcript levels of singlet oxygen-responsive genes occur after UV-B radiation. **Fig. 6** shows that singlet oxygen-responsive genes were significantly up-regulated after UV-B exposure, and that they were also differentially expressed in the *cbs3* and *lut2* mutants in comparison to WT plants, both under control conditions and after UV-B exposure. While *AAA-ATPase* (one of the six AAA-ATPases of the proteasome regulatory particle), *BAP1* (encoding a protein with a C2 domain, involved in defense and cell death regulation) and *FLOT3* (encoding a protein involved in an endocytic pathway; Lee *et al.*, 2007) transcripts were significantly increased after UV-B exposure, levels were lower in *lut2* than in *cbs3* and WT plants, suggesting that oxidative stress (an in particular singlet oxygen stress) produced after exposure is lower in *lut2* plants that accumulate β,β-xanthophylls. To validate this hypothesis, we analyzed the integrity of the membrane cells after UV-B exposure by measuring electrolyte leakage, which is a measure of oxidative damage. WT and *cbs3* plants showed a significant increase in leaf electrolyte leakage after UV-B exposure, with *cbs3* plants showing higher values than WT plants; however, *lut2* plants exhibited no changes in electrolyte leakage after UV-B exposure, with lower values than WT and *cbs3* plants (**Fig. 6A**). In addition, UV-B sensitivity was assayed though lipid peroxidation analysis (Blokhina *et al.,* 2003), by measuring the thiobarbituric acid-reactive substances (TBARS) content. TBARS were increased by UV-B in WT plants, while levels were high and similar under both conditions in *cbs3* mutants (**Fig. 6B**). On the contrary, *lut2* plants showed lower TBARS than WT plants, both under control and UV-B conditions (**Fig. 6B**). Together, our results demonstrate that β,β-xanthophylls protect the plants against oxidative damage after UV-B exposure, probably through protection against singlet oxygen stress. It is possible then that changes in the oxidative status of the cells may affect the expression of not only singlet oxygen-responsive genes (**Fig. 6**); but also DNA repair enzymes (**Fig. 5**).

β,β-xanthophylls can protect against DNA damage after UV-B exposure in vitro

Finally, we analyzed if the role of β,β-xanthophylls in the protection against DNA damage by UV-B can be a consequence of direct absorption of this radiation. Therefore, we *in vitro* analyzed DNA damage after different times of UV-B exposure using DNA purified from WT Arabidopsis plants in the presence or absence of violaxanthin. In addition, DNA damage was also analyzed in the presence of rutin (quercetin 3-O-rutinoside) as a positive control, as quercetin is a UV absorbing compound that has been previously demonstrated to protect against DNA damage (Emiliani *et al.*, 2013). **Fig. 7** shows that after 5, 10 and 15 min of UV-B exposure, CPD accumulation increases. However, when DNA is incubated in the presence of rutin or violaxanthin, DNA damage is significantly decreased. On the contrary, when these experiments were done in the presence of other xanthophylls (neoxanthin and β carotene), DNA damage was similar to that irradiated in the absence of any compound (**Fig. S3**). Thus, although these experiments were done *in vitro*, our results suggest that β,β-xanthophylls, and violaxanthin in particular, could directly protect DNA against CPD formation by UV-B.

Discussion

Isoprenoid derivatives are vital for all living organisms; in particular, plant isoprenoids participate in respiration, photosynthesis, membrane fluidity, and in the regulation of growth and development (Vranová *et al.*, 2013). Moreover, as specialized metabolites, they also have important roles in allelopathic and plant-pathogen interactions, and in attraction of pollinators and seed-dispersing animals. In plants, a large group of isoprenoids participate in photosynthetic processes, including light harvesting, energy conversion, electron transfer, and quenching of excited chlorophyll triplets (for a revision see Vranová *et al.*, 2013). In particular, carotenoids (including carotenes and their oxygenated derivatives, the xanthophylls) are isoprenoids that quench excess excitation energy during light harvesting to

protect the light-harvesting complex acting as antioxidants (for a revision, see Munné-Bosch et al., 2013). Carotenoids are lipophilic antioxidants that achieve essential roles in controlling oxidants such as singlet oxygen generated by PSII, when they are generated within the thylakoid membranes. In addition, they also participate in signal transduction pathways; for example, the oxidation of β-carotene leads to the production of signals that regulate gene expression or trigger cell death (Ramel *et al.*, 2012). In this case, the oxidation of β-carotene by singlet oxygen produces different volatile derivatives that regulate the expression of singlet oxygen-responsive genes (Ramel *et al.*, 2012).

In plant plastids, all isoprenoids, including carotenoids, are synthesized by the 5-C units IPP and DMAPP that are produced through the MEP pathway. In this manuscript, we demonstrate that MEP-derived isoprenoid metabolites participate in UV-B protection in plants. First, we here show that after a UV-B treatment, there is an increase in the levels of specific plastidial isoprenoids, such as γ-tocopherol and the β,β-xanthophyll zeaxanthin (**Table 1**). In agreement with this result, plants deficient in the MEP pathway enzymes DXS (*A6*) or HDS (*cbs3*) have increased DNA damage after UV-B exposure and decreased photosynthesis (**Fig. 2**). Interestingly, after the UV-B treatment, *A6* and *csb3* plants show a higher decrease in the maximum efficiency of PSII than WT plants (**Fig. 2B**); this can be a consequence of an increase in oxidative stress in these plants after exposure, due to decreased levels of β-β xanthophylls (**Figs. 5 and 6**). Previously, it was reported that the MEP intermediate MEcPP has an important role during stress responses (Flores-Perez *et al.,* 2008). However, the fact that both *A6* and *cbs3* accumulate lower or higher levels of this metabolite, respectively, but similar levels of CPDs demonstrate that, in our experimental conditions, this metabolite is not playing a major role in UV-B protection.

Second, a thorough genetic approach showed that an imbalance in the xanthophyll levels but not in tocopherol accumulation **(Fig. 3)** triggers a differential impact in CPD accumulation after UV-B exposure. Particularly, we demonstrate that plants that over-accumulate β,β-xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) but lack β,ε-xanthophylls (lutein), show decreased DNA damage after UV-B exposure (**Fig. 4**). Remarkably, *in vitro* assays demonstrate that violaxanthin can protect against DNA damage by UV-B (**Fig. 7**), probably through direct absorbance. Although experiments *in planta* are required, it is possible that β,β-xanthophylls may have an *in vivo* role absorbing UV-B for protection against DNA damage. Despite this, the role of β, β xanthophylls as screen pigments in the UV-B region might be very minor based on their absorption spectra. On the other hand, CPD formation requires a complex singlet/triplet excitation dynamics, where the triplet excited state of the pyrimidine dimer plays a key role before the ground state CPD is finally formed (Zhang and Eriksson, 2006). Therefore, if β, β xanthophylls directly participate

in DNA photoprotection by quenching the singlet/triplet excited state of the pyrimidine dimer to avoid CPD formation, these compounds should be in close vicinity to DNA molecules. The presence of xanthophylls in the nuclei has not been reported; but this possibility cannot be ruled out. Alternatively or in addition, plants that accumulate higher levels of β,β-xanthophylls express higher levels of DNA repair enzymes (**Fig. 5**); hence, increased repair may also be a mechanism that could explain the lower DNA damage observed in *lut2* plants.

ABA levels were previously determined in *aba2* mutants by González-Guzmán *et al.* (2002) and in *A6* transgenic plants in *Estevez et al.* (2001). Despite *A6* transgenic plants with low ABA levels show increased UV-B damage compared to WT plants; *aba2* plants, which also accumulate low ABA, do not show increased DNA damage after exposure. Therefore, we also here provide evidence that this phenotype is specifically generated by the β,β-xanthophylls and is not mediated by their downstream product, the stress-response hormone ABA (Xiong and Zhu, 2003; **Fig. 4)**. Interestingly, despite that *lut2* mutants have significantly higher levels of β,β-xanthophylls that most mutants analyzed in this work (Z+V+A; **Table S3**), they still have 2.4 times lower amounts of these compounds than *aba1*, which show similar DNA damage as WT plants. However, this difference is accounted mostly by zeaxanthin, which has a minor role in UV-B protection compared to violaxanthin + antheraxanthin as shown in the Factor Analysis presented in **Table S5**. According to the data presented here, violaxanthin and antheraxanthin are the most important β,β-xanthophylls to provide DNA damage protection, and *aba1* mutants are deficient in these two compounds. Therefore, despite the fact that zeaxanthin can provide some UV-B shielding, high accumulation of this compound is not enough to protect plants as are high levels of violaxanthin and antheraxanthin.

The β,β-xanthophylls participate in the xanthophyll cycle, which has an important protection role in conditions of excess light energy. Under normal light conditions, the enzyme zeaxanthin epoxidase (ZEP) converts zeaxanthin into violaxanthin, thus maintaining the levels of violaxanthin elevated. However, when light intensity is too high, the enzyme violaxanthin de-epoxidase (VED) is activated, converting violaxanthin back to zeaxanthin (**Fig. 1**). In this way, under high light conditions, plants maintain high zeaxanthin levels, this β,β-xanthophyll is reported to be a better quencher than violaxanthin. Thus, while zeaxanthin has been several times reported to play different photoprotective roles (Horton *et al.*, 1996; Holt *et al.*, 2005; Baroli *et al.*, 2003; Dall´Osto *et al.*, 2006); the function of violaxanthin as a direct photoprotector is more controversial. However, our results provide evidence that under UV-B conditions, not only zeaxanthin, but all β,β-xanthophylls, probably through the xanthophyll cycle, can protect plants against excess exposure.

Previous reports using tobacco transgenic plants overexpressing the *β*-carotene hydroxylase correlated the increased in zeaxanthin levels to a higher tolerance against UV radiation (Götz *et al.*, 2002). In this work, the authors also measured an increase in violaxanthin levels after UV-B exposure in these transgenic plants. In the experiments presented here, the analysis of *aba1*, which does not metabolize zeaxanthin into downstream xanthophylls and accumulates CPDs levels similar to those in WT plants after UV-B exposure, suggest the existence of an active role of violaxanthin and anteraxanthin in UV-B photoprotection. This is further validated by the Factor Analysis presented in **Table S5**. On the other hand, tobacco plants overexpressing Arabidopsis *β-carotene hydroxylase* and accumulating xanthophylls also displayed more tolerance to UV radiation, as shown by less leaf necrosis (Zhao *et al.*, 2013). Interestingly, the authors suggested that, as more xanthophylls were accumulated in these plants, it could be possible that at least some of them may be free from photosystems and biologically active as free pigments, conferring protection under stress conditions. Therefore, violaxanthin may also be responsible of the increased UV tolerance reported in these works, in agreement with our results. Although carotenes do not directly absorb in the UV-B range of light, they have been several times demonstrated to protect against UV-induced photodamage, and they have been known as "sun protectants" (Biesalski and Obermueller-Jevic, 2001). In this way, in animal epidermal cells, carotenoids can protect *in vivo* against UV-B sensitized photochemical reactions, where excited species occur with exposure to UV-B light. Thus, carotenoid pigments have been proposed to have anti-skin cancer activity because they can quench excited species in epidermis exposed to UV-B (Mathews-Roth, 1986).

Carotenoids have been demonstrated to protect against singlet oxygen stress because they have the capacity to quench $^1O_2$ through a physical mechanism involving transfer of excitation energy followed by thermal deactivation; and also by a chemical mechanism involving their oxidation (Ramel *et al.*, 2012). In the presence of $^1O_2$, β-carotene, lutein, and zeaxanthin are oxidized to various aldehydes and endoperoxides, which are rapidly accumulated during high-light stress. This accumulation parallels the degree of photosystem (PS) II photoinhibition and the expression of singlet oxygen marker genes. In our experiments, UV-B radiation also induce the expression of singlet oxygen marker genes and produce damage to membranes, which are reduced in plants accumulating higher levels of β,β-xanthophylls (**Figs. 5** and **6**). In this way, besides affecting the expression of singlet oxygen marker genes, it is also possible that changes in the oxidative status of the cells regulated by β,β-xanthophylls may affect the expression of DNA repair enzymes, and as a consequence DNA damage levels.

It is interesting to note that the upregulation of *PSY* and *VDE* by UV-B correlate with an increase in *HY5* transcript levels, in agreement with previous results from Toledo-Ortiz *et al.* (2014), where they demonstrate that this transcription factor regulates the expression of these 2 genes. Therefore, the increase in zeaxanthin levels after UV-B exposure may be a consequence of activation of metabolic pathways from precursors generated by the MEP pathway regulated by HY5.

Xanthophylls have been previously related to confer tolerance to different stresses. For example, tobacco plants overexpressing lycopene *β*-cyclase or *β*-carotene hydroxylase gene show a significant increase in the levels of xanthophyll cycle pigments without affecting the ABA content, making the plants more tolerant to salt or drought stress, respectively (Zhao *et al.*, 2013; Jin *et al.*, 2015). Moreover, increased zeaxanthin levels by overexpression of *β-carotene hydroxylase* in Arabidopsis have been shown to confer tolerance to high light and high temperature (Davison *et al.*, 2002). These transgenic plants show decreased leaf necrosis and anthocyanin contents, and reduced lipid peroxidation. Interestingly, these plants also overaccumulate violaxanthin (Davison *et al.*, 2002). Therefore, cooperative effects on photoprotection by different xanthophyll species may exist. It is then possible that β,β-xanthophylls may have a similar protective role in plants after UV-B exposure.

Overall, the results presented here demonstrate that Arabidopsis plants accumulating higher levels of β,β-xanthophylls are more tolerant to UV-B damage. Violaxanthin, antheraxanthin and zeaxanthin all participate in the xanthophyll cycle, and our data show that zeaxanthin levels significantly increase after UV-B exposure. Thus, and similarly as it has been demonstrated under excess white light conditions, this cycle could have an important protection role under UV-B.

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Supplementary data

Fig. S1. Transcriptomic changes of MEP, carotenoid biosynthesis and degradation pathways in 4-week-old Arabidopsis thaliana plants after 4h UV-B exposure (1.31 kJ m^{-2}). Data was obtained from Das *et al.* (2016; GSE80111).

Fig. S2. (A-C) Relative expression of flavonoid metabolism enzymes chalcone synthase (*CHS;* A) and flavanone 3-hydroxylase (*F3H;* B) and the UV-B regulated transcription factor *HY5* (C) by RT-qPCR in WT, *aba1* and *aba2* plants under control conditions and after UV-B exposure. Data are represented as the means obtained from biological triplicates; the error bars indicate the S.E. of the samples. (D-G) UV-B-absorbing compounds after UV-B exposure. Total UV-B-absorbing compounds quantified by their absorbance at 312 nm from WT, *aba1* and *aba2* plants (D), and WT and *lut2* plants (E) were assayed after 4 h UV-B or in untreated controls (C). Measurements are the average of six adult leaves from six different plants. Error bars represent the SEM. Statistical significance was analyzed using two-way ANOVA, Tukey test with $P<0.05$; differences from the control are marked with different letters.

Fig. S3. CPD levels in DNA under control conditions (UV-B 0.02 W m-2 for 15 min, C) and after a UV-B treatment (UVB 0.5 W m-2) for 15 min. The DNA was irradiated in the presence of neoxanthin (A), or β carotene, or in the absence of any compound. 2 µg of DNA was loaded in each well. Measurements are the average of six samples. Error bars represent SEM. Different letters denote statistical differences (Tukey test; $P < 0.05$) applying a two-way ANOVA tests using Sigma Stat 3.1.

Table S1. *Transgenic and mutant Arabidopsis plants used in this study.*

Table S2. *Primers used for RT-qPCR.*

Table S3. *Isoprenoid products from the MEP pathway relative to the internal standard canthaxanthin in leaves from Col-0 WT plants and mutants grown in the absence of UV-B and after a 4h UV-B treatment.*

Table S4. *Isoprenoid products from the MEP pathway relative to the internal standard canthaxanthin in leaves from RLD WT plants and A6 transgenic plants in the absence of UV-B and after a 4h UV-B treatment.*

Table S5. *Percentage of variation, percentage of accumulated variation and factor loadings associated to the first two factors after varimax rotation.*

Accession Numbers.

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Table 1. *Isoprenoid products from the MEP pathway relative levels to those in Col-0 control plants. Isoprenoids showing statistical significant differences (P<0.05) to those in Col-0 control plants are shown with an asterisk, in bold are shown those with higher levels than Col-0 control, while those that accumulate at lower levels are underlined. N.D.:* not detected.

	Control					UV-B				
	Col-0	***csb3***	***lut2***	***aba2***	***aba1***	**Col-0**	***csb3***	***lut2***	***aba2***	***aba1***
Violaxanthin	1.00±0.17	0.54±0.10*	**1.80±0.27***	1.01±0.49	N.D.	0.97±0.12	0.60±0.06*	**1.77±0.34***	0.84±0.22	N.D.
Neoxanthin	1.00±0.08	0.90±0.05	0.69±0.07*	1.07±0.02	N.D.	1.04±0.07	1.06±0.07	0.70±0.08*	1.07±0.06	N.D.
Lutein	1.00±0.08	0.83±0.03*	N.D.	1.10±0.11	**1.24±0.11***	0.92±0.08*	0.91±0.05	N.D.	1.09±0.08	**1.12±0.06***
Zeaxanthin	1.00±0.50	1.01±0.06	**11.75±4.16***	1.15±0.11	**438.68±22.33***	**13.05±2.82***	**24.83±1.88***	**15.00±5.77***	**15.77±3.25***	**423.94±25.44***
β-carotene	1.00±0.08	0.70±0.05*	1.08±0.12	1.12±0.09	**1.52±0.35***	0.90±0.10*	0.90±0.06	1.09±0.10	1.08±0.09	**1.31±0.11***
Chlorophyll b	1.00±0.08	0.93±0.03*	0.78±0.08*	1.02±0.02	1.19±0.24	0.94±0.07*	0.87±0.04*	0.80±0.12	0.99±0.05	1.04±0.15
Chlorophyll a	1.00±0.09	0.85±0.04*	0.78±0.10*	1.08±0.03	1.20±0.23	0.93±0.08*	0.83±0.05*	0.83±0.12	0.95±0.07	1.04±0.13
γ-tocopherol	1.00±0.37	0.71±0.07*	0.96±0.37	**1.27±0.19***	**3.18±0.45***	**1.88±0.42***	**1.39±0.12***	1.16±0.43	**1.52±0.38***	**2.42±3.38***
α-tocopherol	1.00±0.15	0.66±0.03*	1.06±0.12	0.91±0.03	**21.24±2.74***	1.03±0.10	0.95±0.06	0.99±0.11	0.94±0.04	**9.71±1.42***
Phytoene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Antheraxanthin	1.00±0.04	0.98±0.05	**7.28±0.67***	1.07±0.02	N.D.	1.06±0.03	1.08±0.09	**7.72±0.34***	1.03±0.05	0.95±0.03

FIGURE LEGENDS:

Fig. 1. (A) MEP pathway in *Arabidopsis thaliana*. Enzymes catalyzing steps regulating different branches are indicated. Deoxyxylulose 5-phosphate synthase (DXS); 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR); 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase (HDS); 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR); phytoene synthase (PSY); phytoene desaturase (PDS); zeta-carotene desaturase (ZDS); carotenoid isomerase (CRTISO); lycopene cyclase (LCY); carotene beta-ring hydroxylase/ oxygen binding (CYP97); carotenoid cleavage dioxygenase (CCD); carotene beta-ring hydroxylase (BCH); zeaxanthin epoxidase (ZEP); violaxanthin de-epoxidase (VDE); 9-cis-epoxycarotenoid dioxygenase (NCED); xanthoxin dehydrogenase (XDH); phytol kinase (VTE5); tocopherol cyclase (VTE1); homogentisate phytyltransferase (VTE2). HY5 regulated steps are named in blue. (B) Relative expression of MEP pathway enzymes under control conditions and after UV-B exposure in Arabidopsis plants. Data are represented as the means obtained from biological triplicates; the error bars indicate the S.E. of the samples. Student's t test with $P<0.05$; differences from the control are marked with asterisks.

Fig. 2. UV-B effects in *A6* and *csb3* plants. (A) CPD relative levels to those in WT plants in DNA of WT (RLD) and transgenic *A6* plants and WT (Col-0) and *csb3* mutant plants under control conditions without UV-B (C) and after a UV-B treatment for 4h. Experiments were done under conditions that allowed photorepair in the light. 2 μg of DNA was loaded in each well. (B) Maximum efficiency of PSII WT (RLD) and transgenic *A6* plants and WT (Col-0) and *csb3* mutant plants under control conditions without UV-B (C) and after a UV-B treatment for 4h. (C) UV-B-absorbing compounds after UV-B exposure. Total UV-B-absorbing compounds were assayed after 4 h UV-B (UV-B) compared with untreated controls (C) WT (RLD) and transgenic *A6* plants and WT (Col-0) and *csb3* mutant plants under control conditions without UV-B (no UV-B) and UV-B treatment for 4h. Measurements are the average of six adult leaves from six different plants. Error bars represent the SEM. Statistical significance was analyzed using ANOVA, Tukey test with $P<0.05$; differences from the control are marked with different letters.

Fig. 3. Tocopherols do not protect Arabidopsis plants against UV-B exposure. CPD relative levels to those in WT plants in DNA of WT (Col-0), *vte1* and *vte2* mutant plants under control conditions without UV-B (C) and after a UV-B treatment for 4h. Experiments were done under

conditions that allowed photorepair in the light. 2 μg of DNA was loaded in each well. Measurements are the average of six adult leaves from six different plants. Error bars represent SEM. Different letters denote statistical differences (Tukey test; $P < 0.05$) applying ANOVA tests using Sigma Stat 3.1.

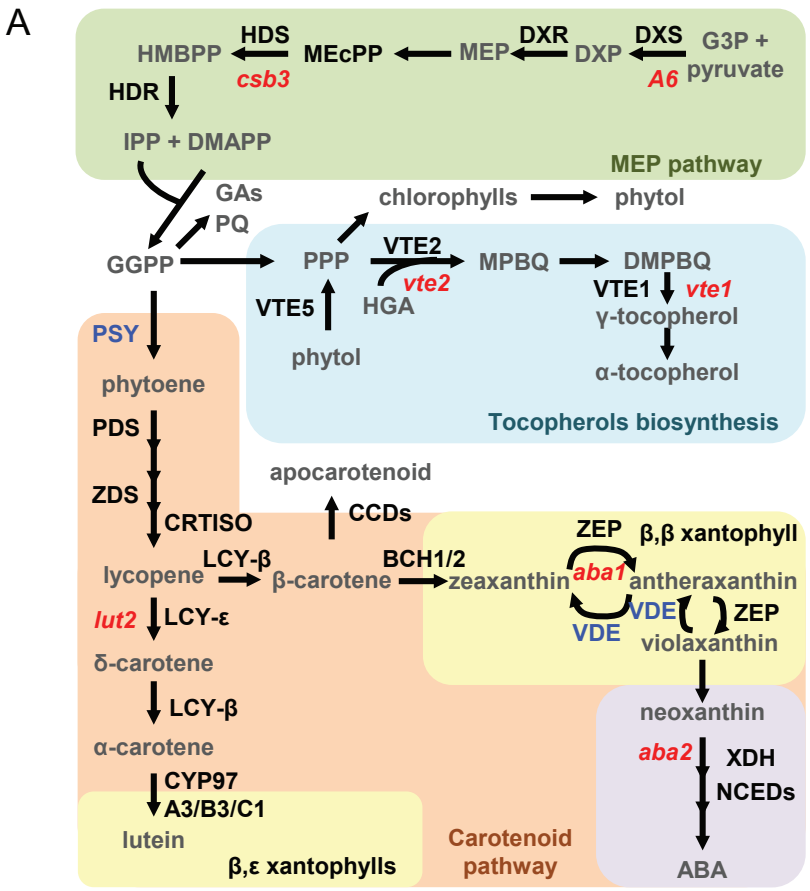
Fig. 4. β,β-xanthophylls protect Arabidopsis plants from UV-B damage. (A) CPD levels in DNA of WT (Col-0) control plants and *lut2* mutant plants under control conditions without UV-B (C) and after a UV-B treatment for 4h. (B) CPD levels in DNA of WT (Col-0) control plants, *aba1* and *aba2* mutant plants under control conditions without UV-B (C) and after a UV-B treatment for 4h. Experiments were done under conditions that allowed photorepair in the light. 2 μg of DNA was loaded in each well. Measurements are the average of six adult leaves from six different plants. Error bars represent SEM. Different letters denote statistical differences (Tukey test; $P < 0.05$) applying ANOVA tests using Sigma Stat 3.1.

Fig. 5. Relative expression of UV-B regulated genes by RT-qPCR in WT, *cbs3* and *lut2* plants. Transcript levels of the *UVR8* photoreceptor (A), the UV-B regulated transcription factor *HY5* (B), the flavonoid metabolism enzymes chalcone synthase (*CHS;* C) and flavanone 3-hydroxylase (*F3H;* D) and the DNA repair photolyase *UVR2* (E) and the DNA repair endonuclease *UVR7* (F) were analyzed under control conditions and after UV-B exposure. Data are represented as the means obtained from biological triplicates; the error bars indicate the S.E. of the samples. Statistical significance was analyzed using a two-way ANOVA test, Tukey test with $P<0.05$; differences are marked with different letters.

Fig. 6. Analysis of membrane injury, lipid peroxidation and singlet oxygen-responsive genes in WT, *cbs3* and *lut2* mutants under control condition or after UV-B treatment. Electrolyte leakage (A) and TBARS content (B) were analyzed under control condition (Control) and after a UV-B treatment (UV-B). Results represent averages ± SE of three independent biological replicates. Statistical significance was analyzed using a two-way ANOVA test at $P < 0.05$; differences are marked with different letters. (C-E) Relative expression of transcripts of singlet oxygen-responsive genes by RT-qPCR. Transcript levels of the *AAA-ATPase* (C), *BAP1* (D) and *FLOT3* (C) were analyzed under control conditions and after UV-B exposure. Data are represented as the means obtained from biological triplicates; the error bars indicate the S.E.

of the samples. Statistical significance was analyzed using ANOVA, Tukey test with $P<0.05$; differences from the control are marked with different letters.

Fig. 7. Violaxanthin prevents UV-B radiation induced CPDs formation in *in vitro* assays. CPD levels in DNA under control conditions (UV-B 0.02 W m^{-2} for 15 min, C) and after a UV-B treatment (UVB 0.5 W m^{-2}) for 5, 10 and 15 min. The DNA was irradiated in the presence of rutin (quercetin 3-*O*-rutinoside, 100 µg mL^{-1}), a flavonol that absorbs UV-B as a positive control, or violaxanthin (14 µg mL^{-1}) or in the absence of any compound. 2 µg of DNA was loaded in each well. Measurements are the average of six samples. Error bars represent SEM. Different letters denote statistical differences (Tukey test; $P < 0.05$) applying a two-way ANOVA tests using Sigma Stat 3.1.



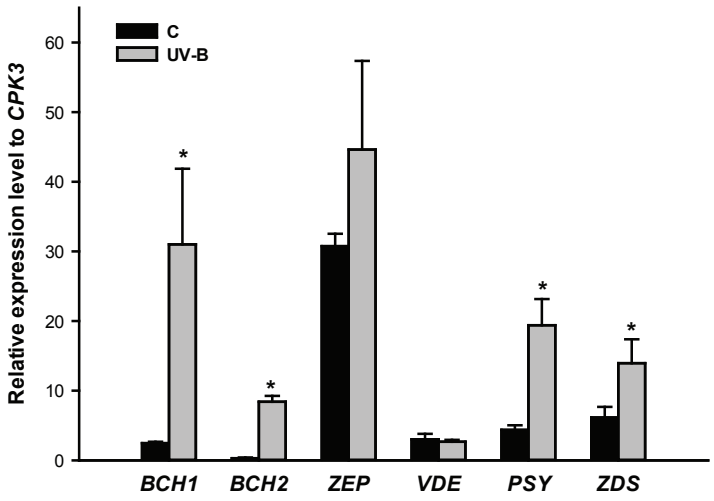


Figure 1

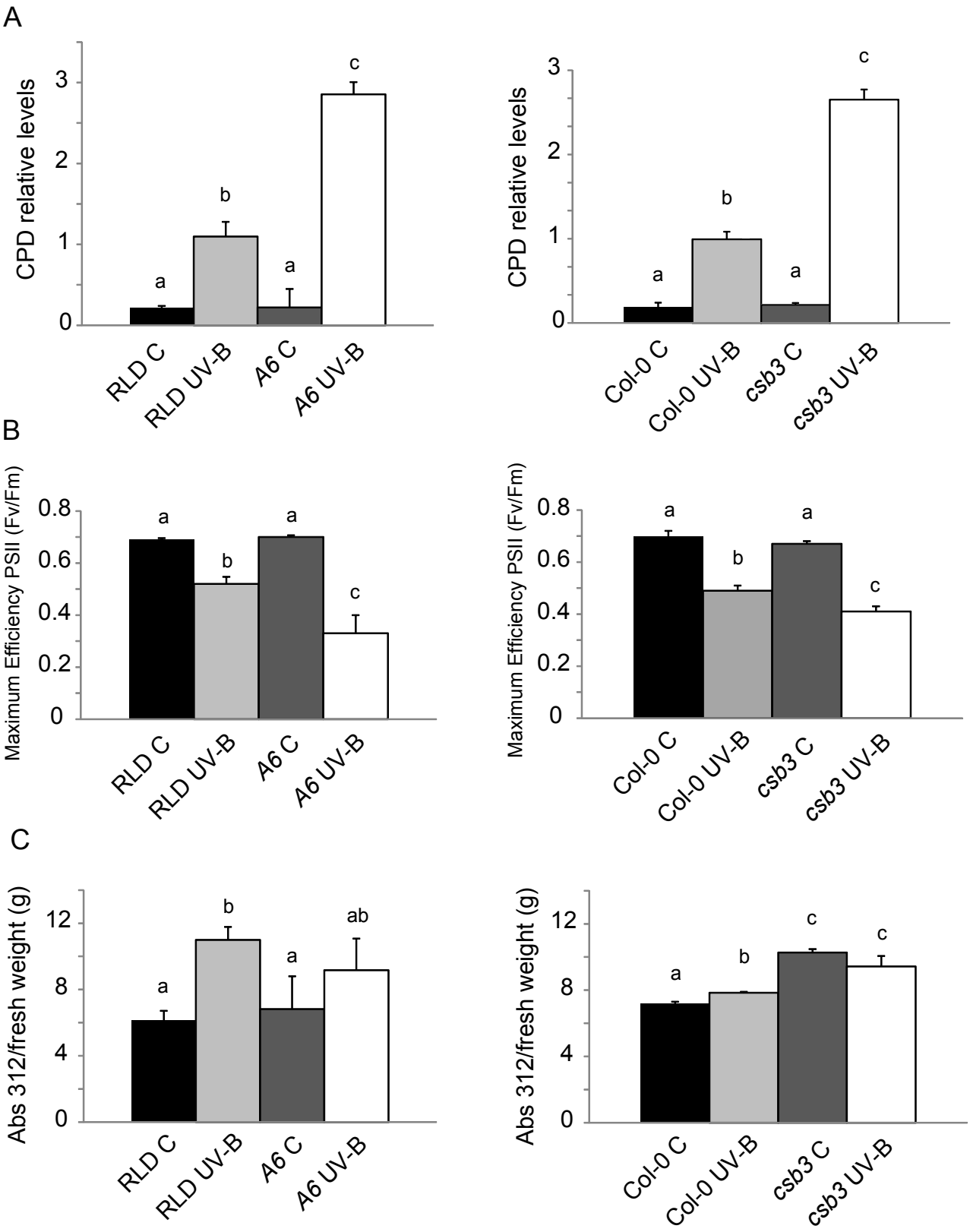
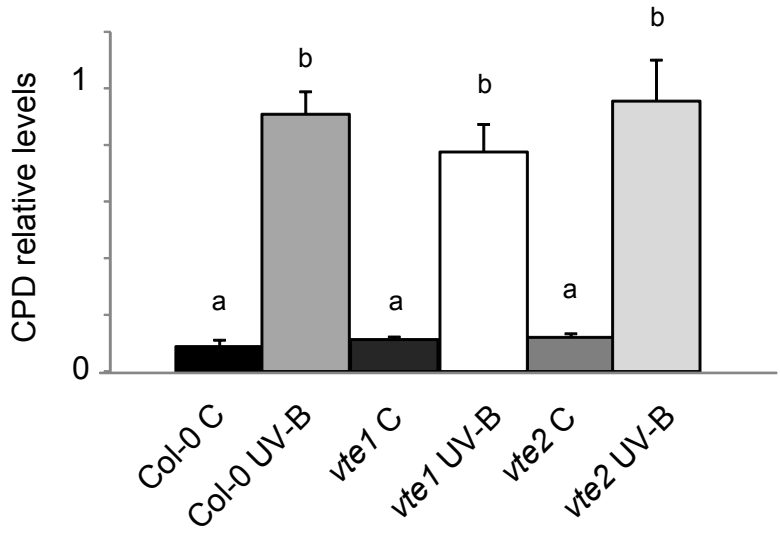


Figure 2



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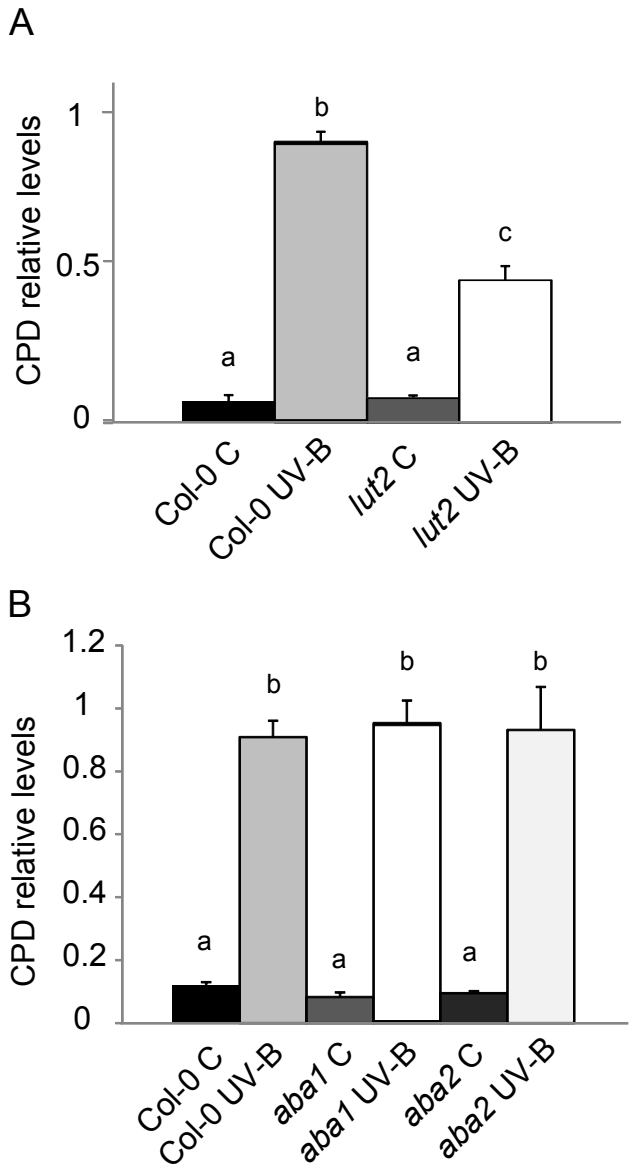

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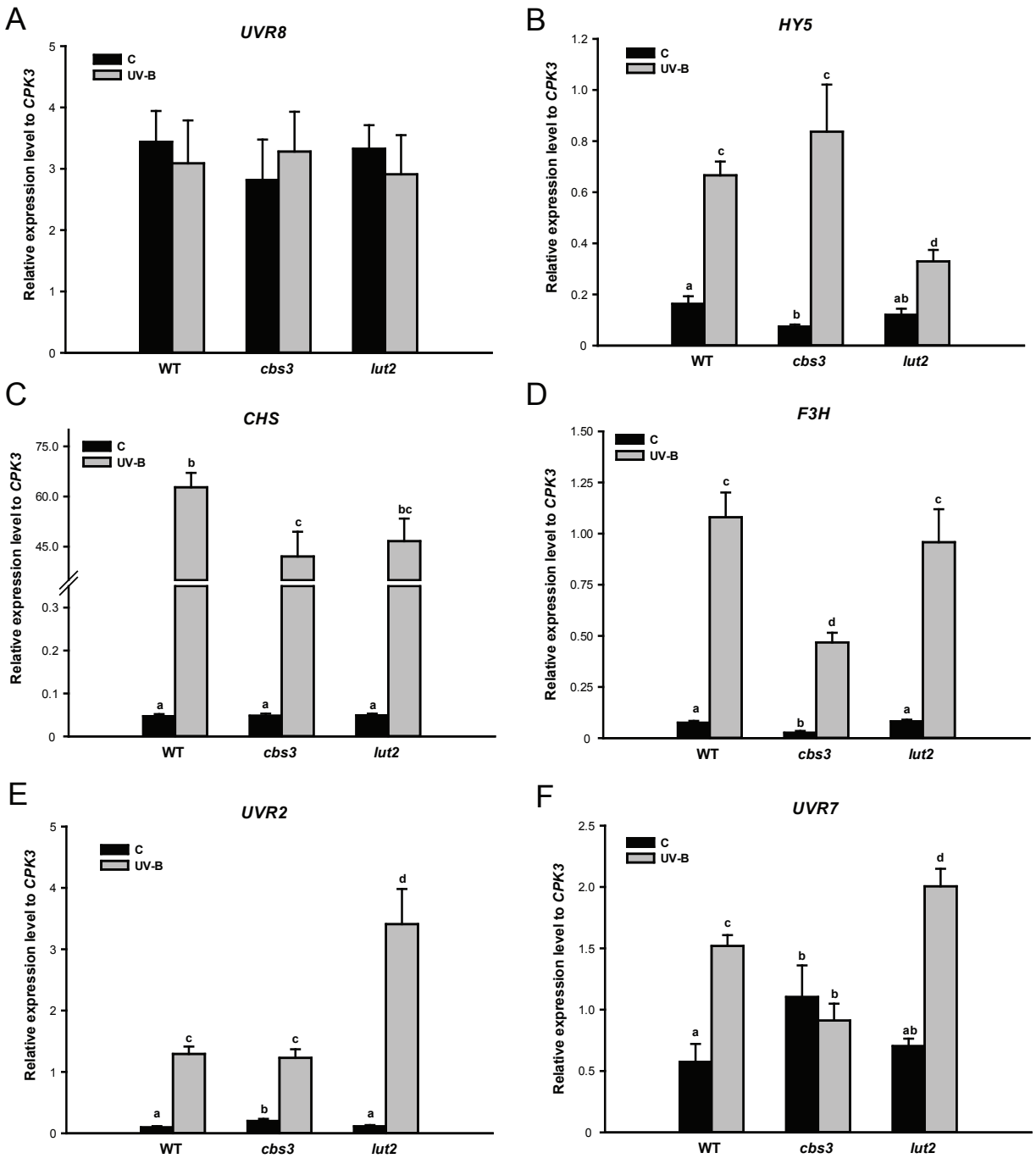

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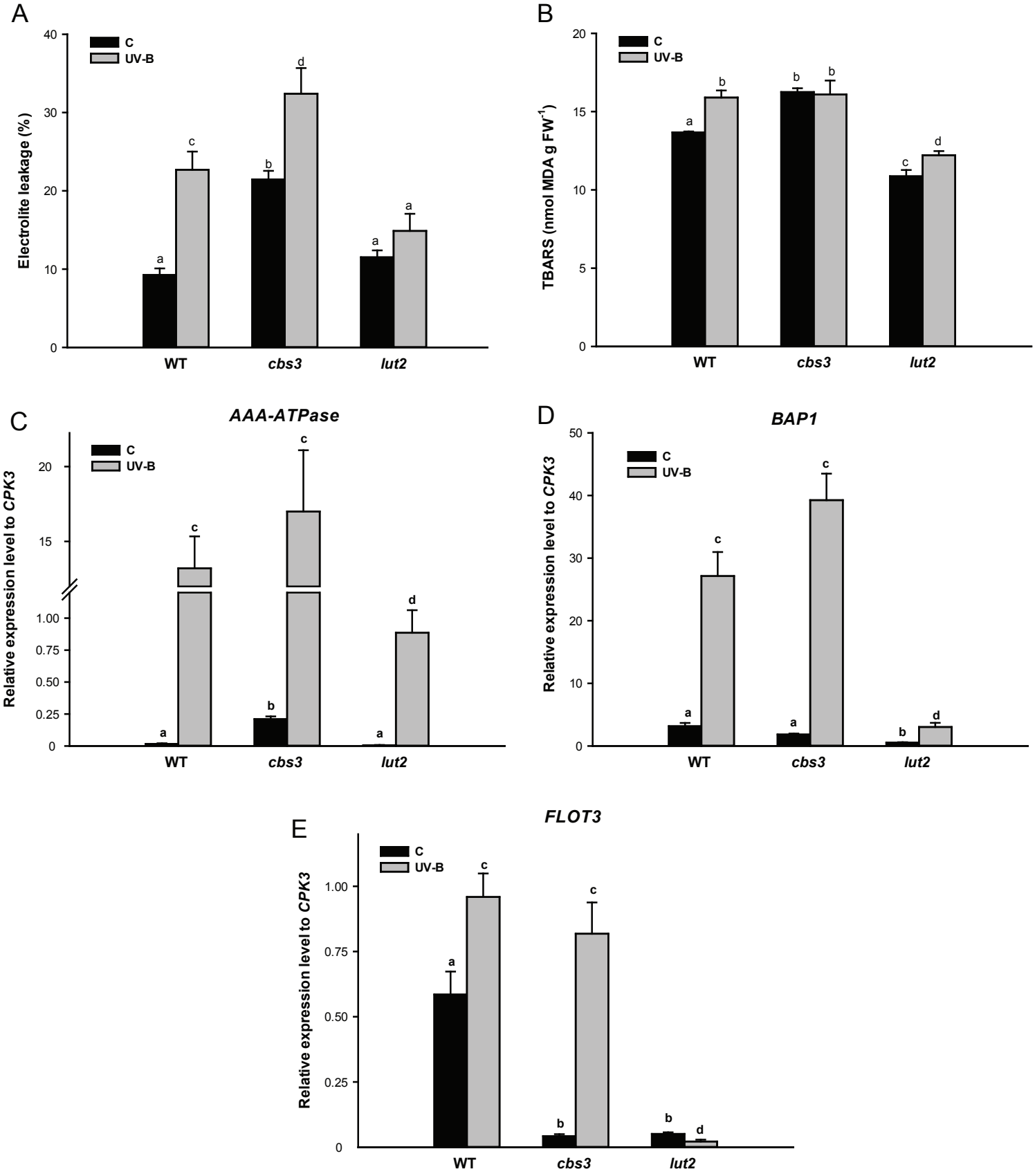

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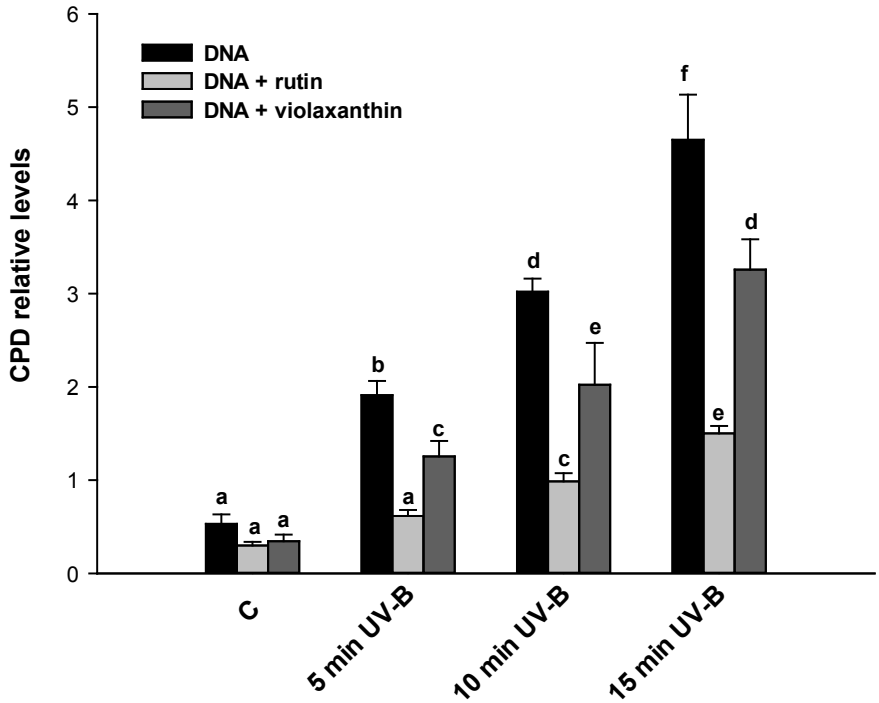

Figure 7